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The use of bulk-tank milk ELISAs to assess the spatial distribution of *Fasciola hepatica*, *Ostertagia ostertagi* and *Dictyocaulus viviparus* in dairy cattle in Flanders (Belgium)

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ABSTRACT

Fasciola hepatica, *Ostertagia ostertagi* and *Dictyocaulus viviparus* are helminth parasites with a wide distribution and an important economic impact in cattle in temperate climates. This paper describes the spatial distribution of *F. hepatica*, *O. ostertagi* and *D. viviparus* in dairy herds in Flanders (Belgium). One thousand eight hundred herds were selected at random from the Flemish dairy population (n = 7002), stratified on community level to obtain a sample representative for the entire study area. From each herd, a bulk milk sample collected in autumn 2006 was analysed with previously described antibody-ELISAs in order to identify herds where the parasite infection level is likely to cause production loss (*F. hepatica* and *O. ostertagi*) (defined as economic infections) or where patent infections have been present over the past grazing season (*D. viviparus*). The herd prevalence of economic infections with *F. hepatica* and *O. ostertagi* was 37.3% (95% Confidence Interval (CI): 35.1–39.7) and 59.1% (95%CI: 56.8–61.4), respectively. The herd prevalence of *D. viviparus* was 19.6% (95%CI: 17.7–21.6). On 28.9% (CI 26.8–31.3) of the herds, low levels of infection were observed for all three of the helminths. The presence of clustering of (economic) infections was studied using Moran's *I*, whereas the location and size of the clusters were studied using the spatial scan statistic, the Local Indicator of Spatial Association and Kernel density plotting. A marked clustering in the spatial distribution of *F. hepatica* and a mild clustering in the spatial distribution of *O. ostertagi* were observed. *D. viviparus* infections were spread evenly over Flanders. Knowledge of locations of high risk areas can lead to increased awareness and may be the start of the development of regionally adapted control measures.

1. Introduction

Fasciola hepatica, *Ostertagia ostertagi* and *Dictyocaulus viviparus* are helminth parasites with a wide distribution in cattle in temperate climates. Previous studies in Western Europe detected herd prevalences of *F. hepatica* in adult dairy cattle of around 50% in England, 86% in Wales (Salimi-Bejestani et al., 2005; Pritchard et al., 2005) and 47% in mixed cattle in Flanders (Lonneux et al., 2002) and *D. viviparus* herd prevalences in adult dairy cattle of around 70% (Eysker et al., 1994; Beugnet et al., 1999), whereas for *O. ostertagi*, all herds with exposure to pasture are considered infected (Agneessens et al., 2000; Charlier et al., 2005b).

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In adult dairy cattle, these helminth infections remain mostly at the subclinical level. Nonetheless, subclinical infections with *F. hepatica* and *O. ostertagi* are considered to be an important cause of reduced productivity (Charlier et al., 2005a, 2007a; Sanchez and Dohoo, 2002). There is no evidence that subclinical *D. viviparus* infections are a cause of important production losses (Vercruysse and Claerebout, 2001), however, since the nineties a shift of clinical outbreaks of *D. viviparus* towards adult dairy cattle has been observed, leading to severe production losses (Ploeger, 2002). Furthermore, anthelmintic treatment costs are an important part of the animal health budget.

The subclinical conditions of most helminth infections in adult cattle hamper diagnosis and cause them to be often overlooked in herd health management programmes. Monitoring the spatial distribution of economically important infections with these helminths using a Geographic Information System will enable to study the presence and location of high risk areas, thus providing possibilities for regionally adapted control measures. The knowledge of the spatial distribution of the helminths can help animal health instances to increase awareness in high risk areas and better target their monitoring and control efforts. This may contribute to a more efficient use of anthelmintics, thus decreasing the treatment costs and limiting the emergence of anthelmintic resistance.

Typically, parasitological studies have been focusing on the prevalence of single parasite infections. However, for efficient helminth control, the farmer benefits more from knowledge of the infection status for all important helminths, so that integrated control measures can be applied. By mapping the spatial distribution of the different important helminths, farmers and veterinarians are provided with this combined knowledge.

With the recent development of tank milk ELISAs for the three helminths (Charlier et al., 2005a, 2007a; Strube et al., 2007), an important tool has been provided for large scale monitoring of the spatial distribution of infections with these three helminths and for identifying herds and areas that could benefit from improved control measures.

The objectives of this paper were to (1) describe the herd prevalence of economic infections with *F. hepatica* and *O. ostertagi* and the herd prevalence of *D. viviparus* in dairy cattle in Flanders and (2) describe the spatial distribution of these infections.

2. Materials and methods

2.1. Study area

The study was conducted in Flanders, Belgium. Flanders has a surface of 13,587 km^2 and a temperate maritime climate. Elevation varies slightly, from sea level in the west to 150 m above sea level in the south and east. Large areas, 24.3% of the total surface, are prone to flooding (Van Orshoven, 2001). In 2006, 7002 dairy farms in Flanders provided milk to a dairy cooperative. The average number of dairy cows per herd in 2006 was 48 (Bernaerts et al., 2008).

2.2. Selection of farms and sample collection

In total, 1800 farms were selected from a list containing all dairy herds in Flanders that provided milk to a dairy cooperative ($n = 7002$). Fourteen hundred farms were randomly selected, stratified on the community level. Additionally, 400 herds were selected randomly from the remaining herds in the list. This procedure was chosen to ensure that the whole study area was covered in the sampling and that 25% of the total study population was sampled. A bulk-tank milk sample was collected from the selected farms in cooperation with the Milk Control Centre Flanders in autumn 2006 (October–November). After arrival at the laboratory (within 72 h after collection), the samples were centrifuged (16,000 $\times$ g for 5 min), the fat was removed and the underlying supernatant was collected and frozen (−20 °C). The samples were kept at 4 °C between collection at the farms and storage at −20 °C.

2.3. Bulk-tank milk ELISAs for the detection of antibodies against F. hepatica, O. ostertagi and D. viviparus

The level of exposure to *F. hepatica*, *O. ostertagi* and *D. viviparus* was determined by antibody-detection ELISAs applied on bulk-tank milk and test results were expressed as an optical density ratio (ODR), which is the measured optical density (OD) of a test sample corrected for the OD of a negative and positive control that were run on each plate. The ELISA for *F. hepatica* was performed as described by Charlier et al. (2007a) and uses excretory–secretory products of *F. hepatica* as antigen. Previously, a negative relationship has been observed between the test results of this ELISA and annual average milk yield, suggesting that the test results can be used to estimate *F. hepatica*-associated milk production losses in a herd. The test results of this ELISA are further referred to as ODRf and a threshold of ODRf > 0.8 was used to identify herds that are likely to suffer production losses because of fasciolosis (Charlier et al., 2007a).

Antibodies against *O. ostertagi* were quantified using the SVANOVIR® *O. ostertagi*-Ab ELISA, following the instructions of the manufacturer. This ELISA uses crude adult worm extract as antigen. This assay measures the exposure of a herd to GI nematodes and identifies herds likely to suffer production losses from subclinical ostertagiosis. The test results are referred to as ODRo and a threshold of ODRo > 0.8 was considered to identify herds likely to suffer production losses caused by ostertagiosis (Charlier et al., 2005a, 2007b).

Antibodies against *D. viviparus* were quantified as described by Von Holtum et al. (2008), adapted for the use of undiluted milk instead of serum. This ELISA uses a recombinant Major Sperm Protein as antigen and detects antibodies raised against patent infections, until 2–6 months post-infection (Fiedor, submitted). As a validation for the use of this ELISA in milk samples of dairy cows, three trials were conducted using groups of 20–23 dairy cows, experimentally infected with 2000 infective lungworm larvae. Faecal and serum samples were taken to confirm the presence of a patent lungworm infection (Strube et al., 2007). Sensitivity and specificity were

calculated to be 100% and 97.5%, using as a cut off ODR 0.49. It remains equivocal whether subclinical *D. viviparus* infections are a cause of important production losses (Vercruysse and Claerebout, 2001) and it is unknown whether the results of this ELISA are correlated to milk production measures. Therefore, this is a qualitative cut off, identifying herds with antibodies against patent *D. viviparus* infection. Test results are further referred to as ODRd.

The *F. hepatica* and *D. viviparus* ELISA have been shown to be highly specific (Von Holtum et al., 2008; De Leeuw and Cornelissen, 1991; Salimi-Bejestani et al., 2005; Schnieder, 1992). The crude antigen used for the *O. ostertagi* ELISA however, is considered to cross-react with antibodies against *Cooperia* species (Keus et al., 1981), and has been shown to cross-react with antibodies against *F. hepatica* and suggested to cross-react with antibodies against *D. viviparus* (unpublished data).

2.4. Data analysis

The selected farms were georeferenced on household location and entered in a Geographic Information System (Manifold System 7x Professional Edition). The intensity and homogeneity of the sample were tested in R 2.8.0 (R Development Core Team, 2008), measuring the number of observations per km^2 and using the Ripley's K function, respectively. The Ripley's K function describes the number of observations within a certain distance of a typical point in the process, and the function of the sample is compared to the normal Poisson distribution (Baddeley, 2008).

Global spatial clustering of ODR values of each parasite was assessed by Moran's *I*. This statistic describes the relationship of a variable in a location with respect to values of this variable in neighbouring locations (Anselin et al., 2006). Moran's *I* was calculated in GeoDA version 0.9.5i (Beta) (https://geoda.uiuc.edu), using a weights file created within GeoDA with a threshold of 5672.5 m (Euclidian distance), assuring a minimum of one neighbour per observation. Statistical significance of the value was calculated by Monte Carlo simulation using 999 iterations.

The location of spatial clusters of 'cases' (herds with ODR above the described cut off) and of 'noncases' was tested using a spatial scan statistic implemented in the software program SaTScan (Kulldorff, 1997; www.satscan.org). Using a circular window, this program can detect high and low rate clusters, containing populations with significantly higher or lower infection rates as compared to the population outside the window. Identification of spatial clusters was done on the assumption of a Bernoulli model. The scan window was set on 50% of the study area and 999 simulations were performed for significance testing.

To confirm the location and size of the clusters, complementary tests were performed. A Kernel's density estimate of the cases was made to visualise areas with a high 'case density'. The Kernel's density maps were produced in R, using the default in the spatstat package, which is an isotropic Gaussian kernel with edge correction (R Development Core Team, 2008).

Moreover, the Local Indicator of Spatial Autocorrelation (LISA) (Anselin et al., 2006) was calculated, identifying herds with ODR values that are strongly spatially autocorrelated. The LISA was calculated in GeoDA using the weights file mentioned for the Moran's *I*.

Because of the expected cross-reactivity of the *O. ostertagi* ELISA with *F. hepatica*, it was investigated if spatial patterns detected for *O. ostertagi* exist independently, or might in fact be caused by patterns in the distribution of *F. hepatica*. To this end, the spatial analysis for *O. ostertagi* was repeated using a dataset excluding all herds with a high level of infection for *F. hepatica*.

3. Results

3.1. Prevalence

Bulk-tank milk samples were collected from 1762 herds. All of these samples were analysed for *F. hepatica* antibodies. In some cases the amount of milk was too small to conduct all analyses, which resulted in 1758 and 1622 test results for *O. ostertagi* and *D. viviparus*, respectively.

The herd prevalence of economic infections with *F. hepatica* and *O. ostertagi* was 37.3% (95%CI: 35.1–39.7) and 59.1% (95%CI: 56.8–61.4), respectively. The herd prevalence of *D. viviparus* was 19.6% (95%CI: 17.7–21.6).

3.2. Spatial distribution

Georeferences were retrieved for 1680 of the selected farms. For *D. viviparus*, 1545 of the farms for which results were obtained were georeferenced. The Ripley's K function for homogeneity confirmed that the sample was evenly spread over Flanders (figure not shown). The intensity of sampling was 0.13 herds/km^2.

The Moran's *I* for *F. hepatica*, *O. ostertagi* and *D. viviparus* was 0.17 ($P < 0.001$), 0.09 ($P < 0.001$) and 0.02 ($P = 0.037$) indicating global clustering of economic *F. hepatica* and *O. ostertagi* infections. Based on the spatial scan statistic in SaTScan, seven significant clusters were found for *F. hepatica*: three (positive) clusters had a higher relative risk compared to the rest of Flanders, of 1.97 (95%CI: 1.74–2.22), 2.22 (95%CI: 1.87–2.62), and 2.73 (95%CI: 2.56–2.90). The other four (negative) clusters had lower relative risks of 0.22 (95%CI: 0.12–0.40), 0.16 (95%CI: 0.068–0.37), 0.08 (95%CI: 0.011–0.54) and 0.34 (95%CI: 0.19–0.60). The *F. hepatica* clusters are shown in Fig. 1 and their characteristics are described in Table 1. The prevalence of economic *F. hepatica* infections in the negative clusters, the area outside the clusters and the positive clusters was 8.4% (95%CI: 5.6–12%), 38% (95%CI: 35–41%) and 70% (95%CI: 64–75%), respectively. Three significant clusters were found for *O. ostertagi*, with relative risks of 1.36 (95%CI: 1.25–1.47), 1.55 (95%CI: 1.40–1.71) and 0.74 (95%CI: 0.67–0.83). The *O. ostertagi* clusters are shown in Fig. 2 and described in Table 2. The prevalence of economic *O. ostertagi* infections in the negative clusters, the area outside the clusters and the positive clusters was 48% (95%CI: 43–52%), 58% (95%CI: 54–61%) and 79% (95%CI: 74–84%), respectively. When the spatial analysis was repeated on the partial dataset (excluding herds with high

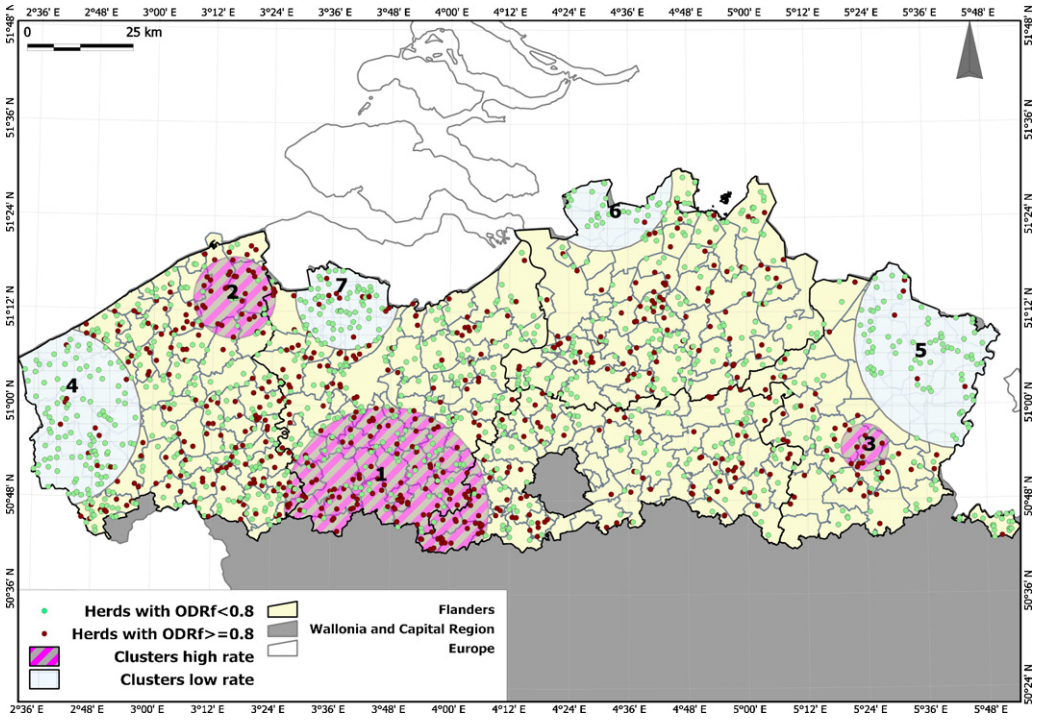

Fig. 1. Clusters detected of high and low prevalence of economic *F. hepatica* infections (bulk-tank milk ODRf ≥ 0.80) in bulk-tank milk samples collected in autumn 2006 on 1680 Flemish dairy herds. Numbers in clusters refer to 'Cluster ID' in Table 1.

Table 1
Clusters of high prevalence of economic *F. hepatica* infections in autumn 2006 on 1680 Flemish dairy herds.

Cluster ID[a]	Population (*n*)	Cases (*n*)	Expected cases (*n*)	Relative risk	95%CI	*P*
1	198	130	73	1.97	1.74–2.22	0.001
2	40	32	15	2.22	1.87–2.62	0.002
3	12	12	5	2.73	2.56–2.90	0.017
4	117	10	43	0.22	0.12–0.40	0.001
5	81	5	30	0.16	0.068–0.37	0.001
6	34	1	13	0.08	0.011–0.54	0.013
7	76	10	28	0.34	0.19–0.62	0.018

Population: total number of samples within the cluster; cases: observed number of samples with a bulk-tank milk ELISA result ≥0.80 ODRf within the cluster; *P*: significance level of the difference in prevalence within and outside of the clusters.
[a] 'Cluster ID' refers to Fig. 1.

infection levels for *F. hepatica*), two significant clusters were found with relative risks of 1.9 (95%CI: 1.67–2.17) and 0.70 (95%CI: 0.59–0.76). The clusters were in the same location as clusters 2 and 3 found in the analysis with the total dataset. The first cluster found in the analysis of the total dataset (see Fig. 2), could not be detected in the analysis with the partial dataset. The clusters of the partial dataset are described in Table 3 and shown in Fig. 3. No clusters were observed for *D. viviparus*.

The Kernel density estimation and the LISA confirmed the location and size of the clusters found for *F. hepatica* and *O. ostertagi* (results not shown).

Table 2
Clusters of high and low prevalence of economic *O. ostertagia* infections in autumn 2006 on 1680 Flemish dairy herds.

Cluster ID[a]	Population (*n*)	Cases (*n*)	Expected cases (*n*)	Relative risk	95%CI	*P*
1	241	186	144	1.36	1.25–1.47	0.001
2	46	42	28	1.55	1.40–1.71	0.014
3	443	211	265	0.74	0.67–0.83	0.001

Population: total number of samples; cases: observed number of samples with a bulk-tank milk ELISA result ≥0.80 ODRo; *P*: significance level of the difference in prevalence within and outside of the clusters.
[a] 'Cluster ID' refers to Fig. 2.

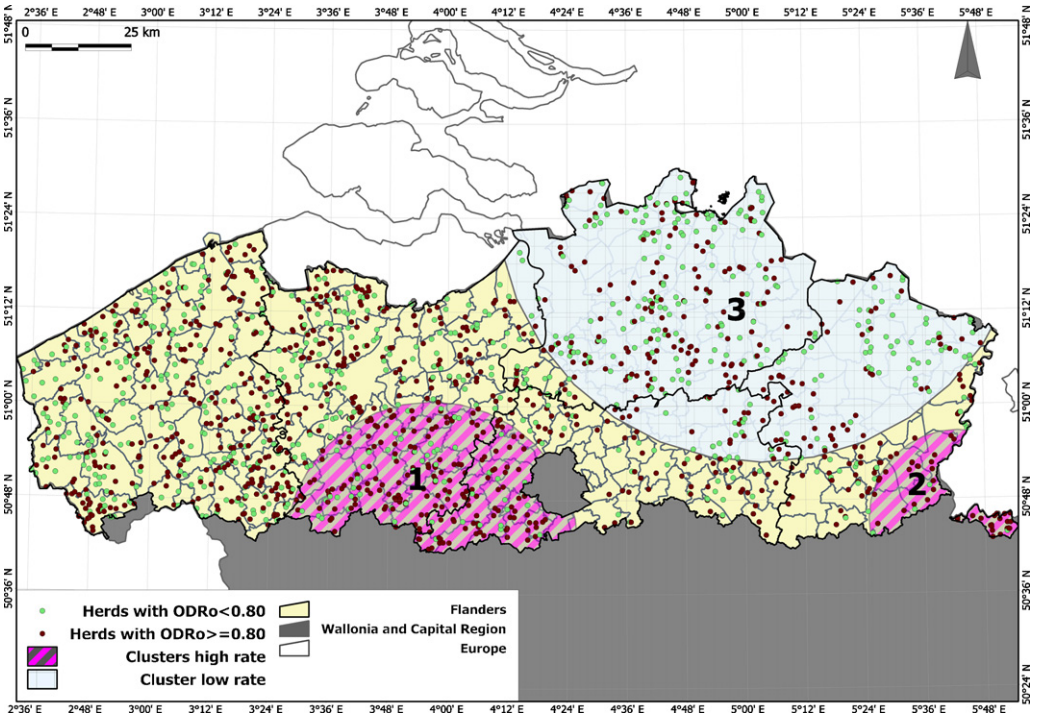

Fig. 2. Clusters detected of high and low prevalence of economic *O. ostertagia* infections (bulk-tank milk ODRo $\geq$ 0.80) in bulk-tank milk samples collected in autumn 2006 on 1680 Flemish dairy herds. Numbers in clusters refer to 'Cluster ID' in Table 2.

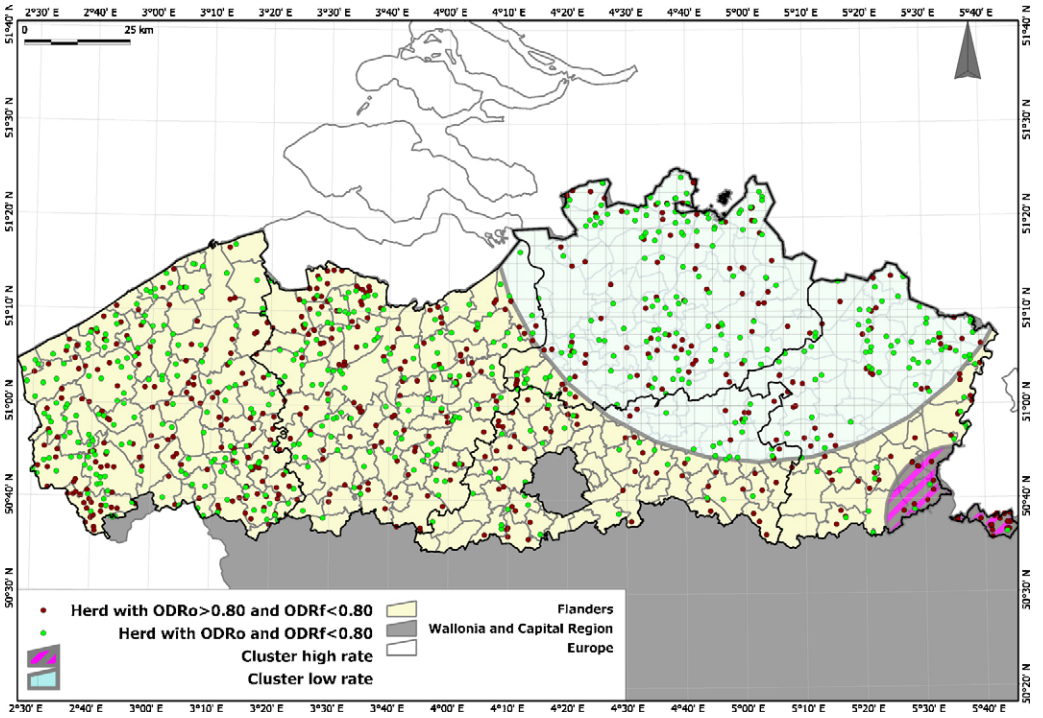

Fig. 3. Clusters detected of high and low prevalence of economic *O. ostertagia* infections (bulk-tank milk ODRo $\geq$ 0.80) in bulk-tank milk samples collected in autumn 2006 on 1055 Flemish dairy herds with ODRf $<$ 0.80.

Table 3
Clusters of high and low prevalence of economic *O. ostertagia* infections in autumn 2006 on 1055 Flemish dairy herds with ODRF < 0.80.

Cluster ID	Population (*n*)	Cases (*n*)	Expected cases (*n*)	Relative risk	95%CI	*P*
Cluster high rate	32	29	15.7	1.90	1.67–2.17	0.006
Cluster low rate	310	116	151.6	0.70	0.59–0.76	0.018

4. Discussion

The present study is one of the first prevalence studies to consider the three most important helminth infections in cattle combined. For *F. hepatica* and *O. ostertagi* this study detected a prevalence of 37.3% and 59.8%, respectively of herds where economic impact of infections is expected, thus identifying herds where improved control methods are advisable instead of merely detecting infection.

The herd prevalence of *D. viviparus* found in this study, 19.6%, is lower than described before in other European countries (Eysker et al., 1994; Beugnet et al., 1999). A possible reason for this is the fact that infections early in the grazing season can be missed if only one sample is taken in October, if an early outbreak was followed by a dry, warm period in which no re-infections occurred. Antibodies in milk can be detected until 2–6 months post-infection (Fiedor, submitted).

The distribution of *F. hepatica* showed a marked spatial pattern. Spatial patterns in distribution of infection can be explained by various factors, such as eco-climatic factors and regional differences in management practices. The more focal distribution of *F. hepatica* as compared to the other two parasites is as expected because of the specific eco-climatic needs of the intermediate host, *Galba truncatula*. It has been shown in several other studies that the prevalence of *F. hepatica* is strongly related to eco-climatic factors (Lonneux et al., 2002; Durr et al., 2005; Pritchard et al., 2005; Ross and Morphy, 1970; Ollerenshaw and Smith, 1969).

This study is the first to show the presence of spatial patterns in the distribution of *O. ostertagi* infections. The locations of the largest clusters observed for *O. ostertagia* and *F. hepatica* show a clear similarity (cluster 1, see Figs. 1 and 2). To investigate if this similarity was due to cross-reactivity of the *O. ostertagi* ELISA with *F. hepatica*, a partial database was analysed (excluding herds with high level of infection for *F. hepatica*). In this analysis, two clusters remain present and the overlapping cluster disappears. These results suggest that the spatial clustering observed in the *O. ostertagi* data, is at least partially due to management, environmental and/or climatic factors. It is generally believed that, within temperate climate zones, the level of infection with *O. ostertagi* depends largely on management factors. However, the free-living stages of *O. ostertagi* and *D. viviparus* also depend on humidity and favourable temperatures for developing and surviving (Eysker, 2002; Gettinby and Paton, 1981; Young et al., 1980a,b). Small changes around the developmental thresholds can have a large impact on population dynamics (Sutherst, 2001). This implies that regional variation in climate, even within small geographical areas, is likely to affect pasture infection levels and exposure of the cows to infective stages of nematodes. There is no definitive answer as to whether the overlapping cluster for *O. ostertagi* and *F. hepatica* is caused by cross-reactivity and/or by correlation between infection status because of overlapping risk factors in management and climate. Further study of the spatial distribution of *O. ostertagi* demands the development of a more specific ELISA.

The absence of clustering in the spatial distribution of *D. viviparus* might be caused by the time of sampling as indicated earlier: infections with *D. viviparus* earlier in the grazing season might be missed because of the decline in antibody levels, as opposed to *F. hepatica* and *O. ostertagi* where the highest level of infection is expected at the end of the grazing season. Further research should be conducted to investigate if with more sampling moments per year, spatial clustering can be detected. Also, only patent infections are measured. Factors that prevent the infections from reaching patency (immunity, treatment), will hide the presence of lungworms in a herd. What is measured is therefore not the presence of lungworm alone, but the presence of lungworm combined with susceptible dairy cows.

After identification of risk areas and risk factors for the presence of (economic) infections, regionally adapted control methods may be developed. The results of this study suggest that for *D. viviparus* this might not be feasible, since no high risk areas were detected. For *O. ostertagi* some clustering has been detected, although the prevalence is high in low, medium and high risk areas (prevalence of 48%, 58% and 79%, respectively). Therefore, for *O. ostertagi* it seems advisable in all areas to always monitor the level of infection using tank milk ELISA and develop a control strategy based on the results. For *F. hepatica*, clear risk areas have been pointed out. In high and medium risk areas (the positive clusters and area outside of the clusters), animal health instances can focus on increasing awareness of farmers and veterinarians of the risk of fasciolosis and on monitoring herd infection levels, e.g. by ELISA and faecal samples. For example, treatment of dry cows and yearlings and management factors such as fencing off risk areas in parcels could be advised in case of high infection levels. In low risk areas diagnosis should only be made when the herd has a history of liver fluke or when, based on location of pastures or symptoms, problems due to liver fluke are suspected.

Finally, because of the dependence of the studied helminths on climate and environment, it is expected that the recent and future climate changes (Crowlye, 2000; IPCC, 2007; Wuebbles et al., 1999) will affect their epidemiology and distribution and alter the infection risks (Van Dijk et al., 2008). Our study results can serve as a baseline from which (economic) prevalences and spatial distributions can be further monitored and associations with eco-climatic factors can be investigated.

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